

DECLARATION

In the matter of an Application for Letters Patent by LTT
BIO-PHARMA CO., LTD.,

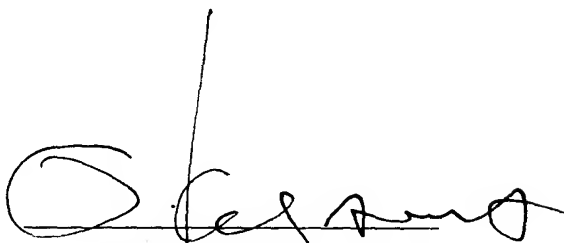
I, Osamu KUSAMA, Patent Attorney, whose full post office
address is 7th Floor, Iwata Bldg., 5-12, Iidabashi 4-chome,
Chiyoda-ku, Tokyo 102-0072, Japan, do solemnly and sincerely
declare as follows:

1. I am well acquainted with Japanese and English language.
2. The following is the true translation into English language
of the Japanese patent application No. JP2004-019439 filled by
LTT BIO-PHARMA CO., LTD. with the Receiving Office / The Japanese
Patent Office on January 28, 2004 in respect of an Application
for Letters Patent.

And I make this solemn declaration conscientiously
believing the same to be true.

Declared at Tokyo, Japan

This 9th day of July, 2006.


Osamu KUSAMA, Ph.D.
KUSAMA PATENT OFFICE

DECLARATION

In the matter of an Application for Letters Patent by LTT
BIO-PHARMA CO., LTD.,

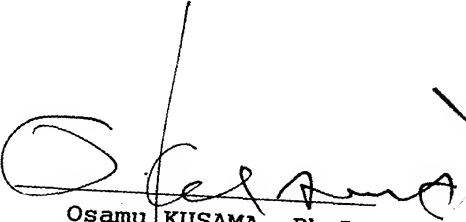
I, Osamu KUSAMA, Patent Attorney, whose full post office
address is 7th Floor, Iwata Bldg., 5-12, Iidabashi 4-chome,
Chiyoda-ku, Tokyo 102-0072, Japan, do solemnly and sincerely
declare as follows:

1. I am well acquainted with Japanese and English language.
2. The following is the true translation into English language
of the Japanese patent application No. JP2004-019439 filled by
LTT BIO-PHARMA CO., LTD. with the Receiving Office / The Japanese
Patent Office on January 28, 2004 in respect of an Application
for Letters Patent.

And I make this solemn declaration conscientiously
believing the same to be true.

Declared at Tokyo, Japan

This 9th day of July, 2006.


Osamu KUSAMA, Ph.D.
KUSAMA PATENT OFFICE

DECLARATION

In the matter of an Application for Letters Patent by LTT
BIO-PHARMA CO., LTD.,

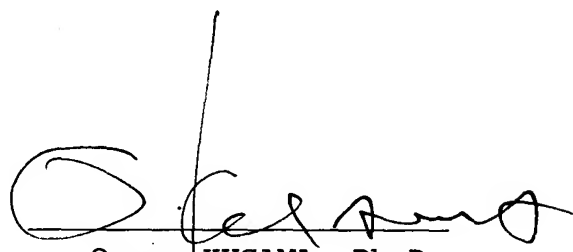
I, Osamu KUSAMA, Patent Attorney, whose full post office
address is 7th Floor, Iwata Bldg., 5-12, Iidabashi 4-chome,
Chiyoda-ku, Tokyo 102-0072, Japan, do solemnly and sincerely
declare as follows:

1. I am well acquainted with Japanese and English language.
2. The following is the true translation into English language
of the Japanese patent application No. JP2004-019439 filled by
LTT BIO-PHARMA CO., LTD. with the Receiving Office / The Japanese
Patent Office on January 28, 2004 in respect of an Application
for Letters Patent.

And I make this solemn declaration conscientiously
believing the same to be true.

Declared at Tokyo, Japan

This 9th day of July, 2006.


Osamu KUSAMA, Ph.D.
KUSAMA PATENT OFFICE

JP2004-019439

(Translation)

JAPAN PATENT OFFICE

5 This is to certify that the annexed is a true copy of the
following application as filed with this office.

Date of Application: January 28, 2004

10 Application Number: Patent Application No. 2004-019439
[ST.10/C]: [JP2004-019439]

Applicant(s): LTT BIO-PHARMA CO., LTD.

15

20

December 27, 2004

25

Hiroshi OGAWA
Commissioner, Japan Patent Office

[Name of Document] Application for Patent

[Reference No.] LTT416

[Addresses] To Commissioner of the Patent Office

[Inventor]

5 [Address] Angelique Okuda 202, 9-11, Okudanishimachi,
 Okayama-shi, Okayama

 [Name] MIZUSHIMA, Toru

[Inventor]

 [Address] 6-12-3-2402, Roppongi, Minato-ku, Tokyo

10 [Name] MIZUSHIMA, Yutaka

[Applicant for the Patent]

 [Identification No.] 303010452

 [Name] LTT BIO-PHARMA CO., LTD.

[Representative]

15 [Identification No.] 100083301

 [Patent Attorney]

 [Name] Osamu Kusama

[Charge]

 [Account Number] 053958

20 [Total Amount] ¥21,000

[List of the Documents]

 [Item] Claims 1

 [Item] Specification 1

 [Ttem] Drawing 1

25 [Item] Abstract 1

 [General Power of Attorney No.] 0313604

【Name of the Document】 CLAIMS

【Claim 1】 A method for screening for compounds safe for gastric mucosa, comprising:

preparing liposome serving as a cell membrane model that is
5 formed of a phospholipid and encapsulates calcein, a fluorescent dye;

allowing a test compound to react with the liposome; and
evaluating the leakage of calcein from the liposome.

【Claim 2】 The method for screening according to claim 1, wherein
10 the calcein leakage is determined by measuring fluorescence at 520 nm.

【Claim 3】 The method for screening according to claim 1 or 2,
wherein the phospholipid for use in the cell membrane model is
selected from the group consisting of phosphatidylcholine,
15 phosphatidylglycerol, phosphatidylserine, phosphatidylinositol,
phosphatidylethanolamine, and cardiolipin.

【Claim 4】 The method for screening according to any of claims 1
to 3, wherein the test compound is an anti-inflammatory compound.

【Claim 5】 The method for screening according to claim 4, wherein
20 the anti-inflammatory compound is a nonsteroidal anti-inflammatory compound or a steroid compound.

【Claim 6】 An anti-inflammatory compound safe for gastric mucosa,
obtained by the method for screening according to claim 4 or 5, or a
salt thereof.

25 【Claim 7】 A liposome serving as a cell membrane model for use in
the screening of compounds having membrane toxicity to gastric
mucosa, the liposome being formed of a phospholipid and
encapsulating calcein, a fluorescent dye.

【Claim 8】 The liposome according to claim 7, wherein the
30 phospholipid for use in the cell membrane model is selected from the
group consisting of phosphatidylcholine, phosphatidylglycerol,

JP2004-019439

phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine,
and cardiolipin.

【Name of the Document】 SPECIFICATION

【Name of Invention】 METHOD FOR SCREENING FOR NONSTEROIDAL
ANTI-INFLAMMATORY DRUGS SAFE FOR GASTRIC MUCOSA

【Technical field】

5 【0001】

 The present invention relates to a method for screening for
compounds or salts thereof that are safe for gastric mucosa and
causes little gastrointestinal side effects. More particularly, the
present invention relates to a method for screening for anti-
10 inflammatory compounds or salts thereof.

 【BACKGROUND ART】

 【0002】

 Of different anti-inflammatory drugs, nonsteroidal anti-
inflammatory drugs (NSAIDs) are known to be particularly effective
15 in reducing inflammation. However, the ability of these drugs to
cause gastrointestinal damage and other serious side effects, in
particular, stomach ulcers, poses a problem to the clinical use of
these drugs. Specifically, the use of nonsteroidal anti-inflammatory
drugs (NSAIDs) is considered the primary cause of stomach gastritis,
20 the currently most prevalent type of stomach ulcer.

 More patients are expected to use NSAIDs with the progress of
aging society. In fact, as the population ages, NSAIDs have become
more widely used to alleviate the symptoms of lumbago, osteoporosis,
rheumatoid arthritis, and other diseases.

25 Thus, the development of NSAIDs that cause no gastrointestinal
side effects is an important task. Such NSAIDs are safe for gastric
mucosa and have less risk of inducing stomach ulcers.

 【0003】

 The present inventors have previously discovered that the
30 ability of NSAIDs to induce stomach ulcers results from the direct
cytotoxicity of NSAIDs and have proposed that NSAIDs lacking direct

cytotoxicity can serve as useful drugs that are safe for gastric mucosa (Patent Document 1).

[Patent Document 1] Japanese Patent Laid-Open Publication No. 2003-207507

5 [0004]

NSAIDs act by inhibiting cyclooxygenase activity in the cell membrane, thereby decreasing prostaglandins that protect gastric mucosa. Also, there have been reports suggesting that some NSAIDs exhibit direct cytotoxicity by inducing necrosis and/or apoptosis.

10 In consideration of these observations, we hypothesized that NSAIDs that inhibit cyclooxygenase activity and do not exhibit direct cytotoxicity can serve as anti-inflammatory drugs that do not cause side effects including stomach ulcers. Based on this hypothesis, we have proposed a screening method that is based on the cytotoxicity
15 to gastric mucosal cells and the inhibition of cyclooxygenase in gastric mucosa.

[0005]

Our further study based on the hypothesis has led to a new discovery that the membrane toxicity of NSAIDs is responsible for
20 their direct cytotoxicity. We have also succeeded in establishing a cell membrane model that allows easy detection of membrane toxicity of a given NSAID. Thus, this cell membrane model can be used to determine if a given NSAID has membrane toxicity and to search for NSAIDs that are safe for gastric mucosa.

25 Our cell membrane model can also be used to search for drugs other than NSAIDs that are safe for gastric mucosa, namely, for mucosa-protecting compounds.

[DISCLOSURE OF THE INVENTION]

[PROBLEMS TO BE SOLVED BY THE INVENTION]

30 [0006]

Accordingly, it is an object of the present invention to

provide a method for screening for compounds or salts thereof --in particular, nonsteroidal anti-inflammatory compounds or salts thereof-- that are safe for gastric mucosa and cause little gastrointestinal side effects.

5 [MEANS TO SOLVE THE INVENTION]

[0007]

To achieve the foregoing object, the essential aspect of the present invention is a method for screening for compounds safe for gastric mucosa, comprising:

10 preparing liposome serving as a cell membrane model that is formed of a phospholipid and encapsulates calcein, a fluorescent dye;

allowing a test compound to react with the liposome; and
evaluating the leakage of calcein from the liposome.

15 [0008]

Specifically, the present invention provides the method for screening, wherein the calcein leakage is determined by measuring fluorescence at 520 nm, and wherein the phospholipid for use in the cell membrane model is selected from the group consisting of

20 phosphatidylcholine, phosphatidylglycerol, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, and cardiolipin.

[0009]

More specifically, the present invention provides the method for screening, wherein the test compound is an anti-inflammatory
25 compound, and the anti-inflammatory compound is a nonsteroidal anti-inflammatory compound or a steroid compound, and further provides an anti-inflammatory compound safe for gastric mucosa, obtained by said method for screening, or a salt thereof.

[0010]

30 Another aspect of the present invention provides a liposome that serves as a cell membrane model for use in the screening method

of the present invention. Specifically, this aspect comprises the liposome serving as a cell membrane model for use in the screening of compounds having membrane toxicity to gastric mucosa, the liposome being formed of a phospholipid and encapsulating calcein, a
5 fluorescent dye. More specifically, the present invention provides the liposome, wherein the phospholipid for use in the cell membrane model is selected from the group consisting of phosphatidylcholine, phosphatidylglycerol, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, and cardiolipin.

10 [EFFECT OF THE INVENTION]

[0011]

The screening method of the present invention relies on a simple cell membrane model to detect membrane toxicity of a given drug. Thus, the screening method of the present invention can be
15 used to easily determine if a test compound, in particular a nonsteroidal anti-inflammatory drug (NSAID), has membrane toxicity. As a result, NSAIDs can be obtained that are safe for gastric mucosa and cause no gastrointestinal side effects. Such NSAIDs are of significant clinical importance.

20 [BEST MODE FOR CARRYING OUT THE INVENTION]

[0012]

The cell membrane model for use in the present invention comprises liposome formed of a phospholipid and encapsulating calcein, a fluorescent dye that permeates the cell membrane. Since
25 the membrane toxicity of NSAIDs is believed to be largely attributed to the disrupted barrier function of the lipid bilayer of cell membrane, by preparing a liposome formed of a phospholipid, encapsulating calcein, a fluorescent dye in the liposome, and allowing NSAIDs to react with the liposome, it is possible to
30 determine the membrane toxicity of a given NSAID by measuring the leakage of calcein from the liposome in the presence of the NSAID.

[0013]

Calcein is a fluorescent dye widely used in the determination of cytotoxicity. Calcein can stain living cells and has no cytotoxicity itself. Further, the examples of the phospholipids to form liposome membrane include phosphatidylcholine, phosphatidylglycerol, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, and cardiolipin. One or more of these phospholipids may be used to form liposome membrane.

[0014]

Thus, the present invention uses the means for determining the degree of damage of phospholipid liposome membrane when it is allowed to react with a given test compound, and in other words, uses the phospholipid liposome membrane as an analogue of gastric mucosa.

Therefore, an important issue herein is if there is a certain correlation between the phospholipid liposome membrane and gastric mucosa. The present inventors have demonstrated that the cytotoxicity of NSAIDs due to necrosis/apoptosis as measured by the decrease in cell viability is well-correlated with the membrane toxicity determined by the liposome model.

[0015]

The present inventors have also demonstrated that the membrane toxicity of NSAIDs is responsible for the direct cytotoxicity of NSAIDs. Specifically, the cytotoxicity of 10 clinically used NSAIDs as measured by the decrease in cell viability (i.e., cytotoxicity due to necrosis/apoptosis as measured by the decrease in cell viability) was compared to their membrane toxicity determined by the liposome model. The results shown in Figs. 1 and 2 revealed a high consistency between the cytotoxicity and the membrane toxicity, indicating the direct cytotoxicity of NSAIDs is attributed to their membrane toxicity. In other words, NSAIDs with strong cytotoxicity

show strong membrane toxicity and NSAIDs with weak cytotoxicity show weak membrane toxicity (Correlation coefficient = 0.91).

[0016]

As described above, the cell membrane model of the present invention using liposome allows the determination of membrane toxicity, and thus the cytotoxicity, of NSAIDs. The present inventors have previously discovered that the ability of NSAIDs to induce stomach ulcers is caused by their direct cytotoxicity and have hypothesized that if a given NSAID is proven to lack direct cytotoxicity in the cell membrane model, that NSAID must be safe for gastric mucosa. Since the direct cytotoxicity of NSAIDs results from their membrane toxicity, it is deduced that NSAIDs can be screened for the inability to damage the gastric mucosa by measuring their membrane toxicity in the cell membrane model provided by the present invention.

[0017]

The screening method of the present invention that screens for NSAIDs that are safe for gastric mucosa by means of the cell membrane model is specifically carried out in the following manner. First, liposome is prepared that is formed of a phospholipid and encapsulates a fluorescent dye such as calcein. NSAIDs to be screened are then allowed to react with the liposome at different concentrations and the leakage of calcein from the liposome (due to the damage to the liposome membrane) is monitored by measuring the fluorescence at 520 nm.

[0018]

Although the screening method of the present invention has been described primarily in relation to NSAIDs, it should be appreciated that the method can be used to screen not only for NSAIDs, but also for a wide range of compounds that are safe for gastric mucosa and thus have no membrane toxicity.

[Examples]

[0019]

The present invention will now be described with reference to Examples.

5 Example 1: Preparation of calcein-encapsulating liposome

Phosphatidylcholine (10 μ mol, 7.7 mg) obtained from egg yolk was dissolved in a 1:2 mixture of chloroform/methanol and the solution was dried. The resulting residue was dissolved in 1.5 mL diethylether and the solution was mixed with 1 mL of a 100mM aqueous
10 solution of calcein-sodium hydroxide (pH 7.4). Diethylether was removed to obtain a solution of liposome encapsulating calcein.

[0020]

Example 2: Calcein leakage from liposome in the presence of different known NSAIDs

15 The following clinically used known NSAIDs were used: indomethacin, ibuprofen, ketoprofen, diclofenac, flurbiprofen, mefenamic acid, flufenamic acid, celecoxib, etodolac, and nimesulide. Each NSAID was added at different concentrations to the liposome solution obtained in Example 1 and the mixture was incubated at 30°C
20 for 10 min. Subsequently, the fluorescence at 520 nm was measured to determine the calcein leakage from the liposome as a measure of membrane toxicity.

The results are shown together in Fig. 3 (for indomethacin, ibuprofen, ketoprofen, diclofenac, and flurbiprofen) and Fig. 4 (for
25 mefenamic acid, flufenamic acid, celecoxib, etodolac, and nimesulide).

[0021]

For indomethacin, ibuprofen, ketoprofen, diclofenac, and flurbiprofen to serve as NSAIDs shown in Fig. 3, the cytotoxicity to
30 gastric mucosal cells decreases in the order of indomethacin > diclofenac > flurbiprofen > ibuprofen > ketoprofen. As can be seen,

the calcein leakage from the liposome decreased in the same order. This supports the hypothesis that nonsteroidal anti-inflammatory compounds that are safe for gastric mucosa can be effectively selected by measuring the calcein leakage from the liposome (*i.e.*, membrane toxicity) observed for the compounds.

[0022]

For mefenamic acid, flufenamic acid, celecoxib, etodolac, and nimesulide to serve as NSAIDs shown in Fig. 4, the cytotoxicity to gastric mucosal cells decreases in the order of celecoxib >

mefenamic acid > flufenamic acid > nimesulide > etodolac. As can be seen, the calcein leakage from the liposome decreased in the same order. Similar to the results shown in Fig. 3, this consistency supports the hypothesis that nonsteroidal anti-inflammatory compounds that are safe for gastric mucosa can be effectively selected by measuring the calcein leakage from the liposome (*i.e.*, membrane toxicity) observed for the compounds.

[INDUSTRIAL APPLICABILITY]

[0023]

As set forth, the simple cell membrane model used in the screening method of the present invention enables detection of the membrane toxicity of test compounds, in particular nonsteroidal anti-inflammatory drugs (NSAIDs). The screening method of the present invention has a significant advantage in that it allows the development of NSAIDs and other clinically useful compounds that exhibit little membrane toxicity and are safe for gastric mucosa.

[BRIEF DESCRIPTION OF THE DRAWINGS]

[0024]

[Fig. 1] Fig. 1 is a diagram showing the correlation between the cytotoxicity (necrosis) of 10 NSAIDs as measured by the decrease in cell viability and their membrane toxicity.

[Fig. 2] Fig. 2 is a diagram showing the correlation between

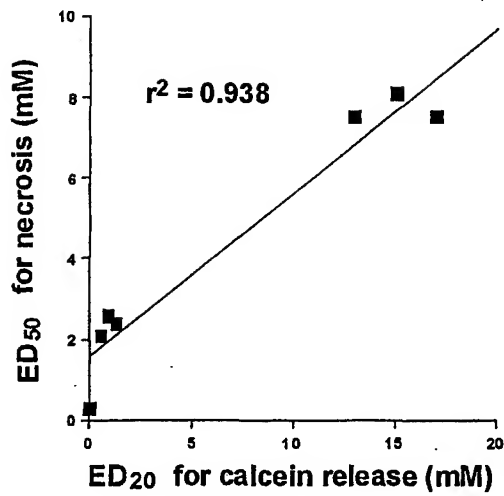
the cytotoxicity (apoptosis) of 10 NSAIDs as measured by the decrease in cell viability and their membrane toxicity.

[Fig. 3] Fig. 3 is a diagram showing some of the results of Example 2 of the present invention.

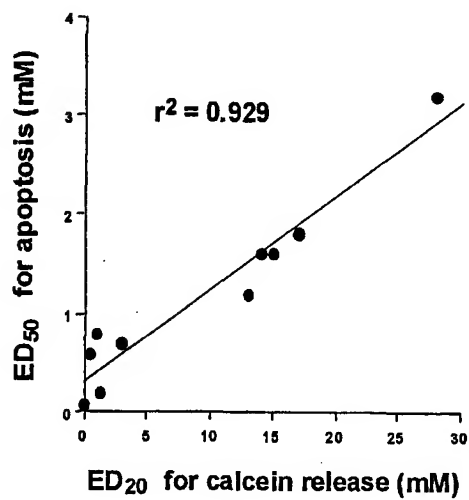
5 [Fig. 4] Fig. 4 is a diagram showing some of the results of Example 2 of the present invention.

[Name of the Document] DRAWING

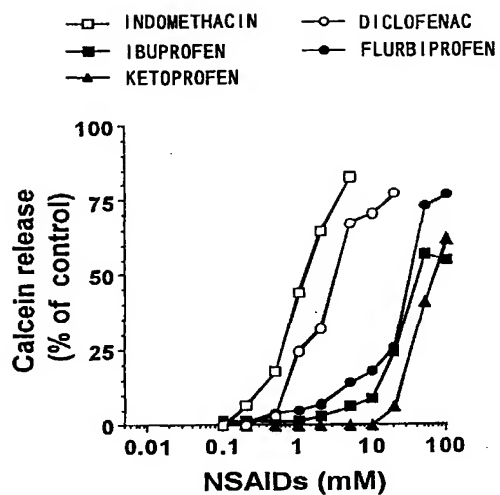
[Fig. 1]



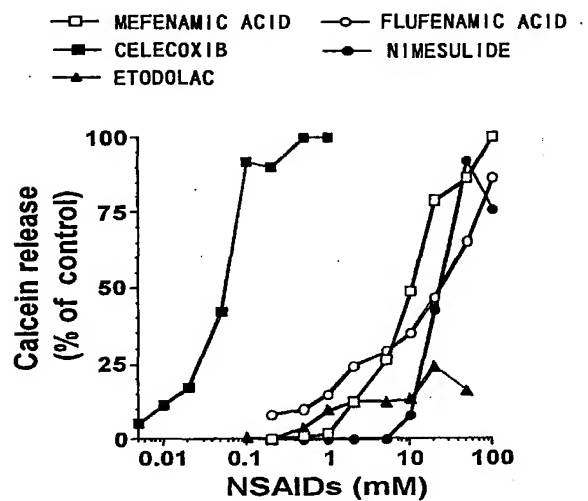
5 [Fig. 2]



[Fig. 3]



[Fig. 4]



[Name of the Document] ABSTRACT

[Abstract]

[Purpose] A method for screening for compounds or salts thereof,
in particular nonsteroidal anti-inflammatory compounds or salts
5 thereof, that are safe for gastric mucosa and cause little
gastrointestinal side effects.

[Means to solve the problem] The method uses a particular
liposome to serve as a cell membrane model. The liposome
encapsulates calcein and is formed of phospholipids, such as
10 phosphatidylcholine, phosphatidylglycerol, phosphatidylserine, and
phosphatidylinositol. A test compound is allowed to react with the
liposome and the leakage of calcein from the liposome is evaluated.
As a result, compounds safe for gastric mucosa, in particular, anti-
inflammatory compounds can be screened.

15 [Selected Figure] Fig. 4

Approval and Addition Information

Patent Application #	Patent Application # 2004-019439
Reference Number	50400138023
5 Name of Document	Application for Patent
Responsible Officer	No.1 Higher Rank 0090
Date	Heisei 16 January 29

<Approval Information and Addition Information >

10	[Date of Submission]	Heisei 16 January 28
----	----------------------	----------------------

Information on the applicant's personal history

Identification Number [303010452]

5	1.	Date of Change	February 25, 2003
		[Reason for Change]	New Registration
		Address	5-1, Atago 2-chome, Minato-ku, Tokyo
		Name	LTT BIO-PHARMA CO., LTD.